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Buddle, Bryce M.; Shu, Dairu; Parlane, Natalie A.; Subharat, Supatsak; Heiser, Axel; Hewinson, R. Glyn; Vordermeier, H. Martin; Wedlock, D. Neil

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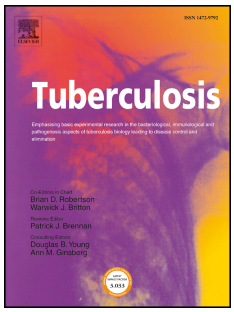

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Bryce M. Buddle, Dairu Shu, Natalie A. Parlane, Supatsak Subharat, Axel Heiser, R. Glyn Hewinson, H. Martin Vordermeier, D. Neil Wedlock

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Vaccination of cattle with a high dose of BCG vaccine 3 weeks after experimental infection with *Mycobacterium bovis* increased the inflammatory response, but not tuberculous pathology

Bryce M. Buddle[a]*, Dairu Shu[a], Natalie A. Parlane[a], Supatsak Subharat[a], Axel Heiser[a], R. Glyn Hewinson[b], H. Martin Vordermeier[b]and D. Neil Wedlock[a]

[a] AgResearch, Hopkirk Research Institute, Grasslands Research Centre, Palmerston North 4442, New Zealand

[b] TB Research Group, Animal and Plant Health Agency, New Haw, Addlestone, Surrey KT15 3NB, United Kingdom

* Author for correspondence. AgResearch, Hopkirk Research Institute, Grasslands Research Centre, Private Bag 11008, Palmerston North 4442, New Zealand. Tel.: +64 6 3518679.

Running title: Effect of BCG vaccination post-*M. bovis* challenge

E-mail addresses: bryce.buddle@agresearch.co.nz (B.M. Buddle), dairu.shu@agresearch.co.nz (D.Shu), natalie.parlane@agresearch.co.nz (N.A. Parlane), art.subharat@agresearch.co.nz (S. Subharat), axel.heiser@agresearch.co.nz (A. Heiser), glyn.hewinson@apha.gsi.gov.uk (R.G. Hewinson), martin.vordermeier@apha.gsi.gov.uk H.M. Vordermeier, neil.wedlock@agresearch.co.nz (D.N. Wedlock)

Summary

A study was undertaken to determine whether BCG vaccination of cattle post-challenge could have an effect on a very early *Mycobacterium bovis* infection. Three groups of calves (n=12/group) were challenged endobronchially with *M. bovis* and slaughtered 13 weeks later to examine for tuberculous lesions. One group had been vaccinated prophylactically with BCG Danish vaccine 21 weeks prior to challenge; a second group was vaccinated with a 4-fold higher dose of BCG Danish 3 weeks post-challenge and the third group, remained non-vaccinated. Vaccination prior to challenge induced only minimal protection with just a significant reduction in the lymph node lesion scores. Compared to the non-vaccinated group, BCG vaccination post-challenge produced no reduction in gross pathology and histopathology, but did result in significant increases in mRNA expression of pro-inflammatory mediators (IFN-γ, IL-12p40, IL-17A, IRF-5, CXCL9, CXCL10, iNOs, and TNF-α) in the pulmonary lymph nodes. Although there was no significant differences in the gross pathology and histopathology between the post-challenge BCG and non-vaccinated groups, the enhanced pro-inflammatory immune responses observed in the post-challenge BCG group suggest caution in the use of high doses of BCG where there is a possibility that cattle may be infected with *M. bovis* prior to vaccination.

KEY WORDS: Bovine tuberculosis; *Mycobacterium bovis*; cattle, vaccination; BCG; vaccine dose

1. Introduction

Bovine tuberculosis (TB) caused predominantly by *Mycobacterium bovis* poses significant economic hardship to livestock farmers as well as constituting a public health problem. It is estimated that >50 million cattle worldwide are infected with *M. bovis*, costing US$3 billion annually [1]. Although, the implementation of "test and slaughter" control programmes has resulted in bovine TB being eradicated from a number of countries [2], these measures have been less effective in countries which have wildlife reservoirs of *M. bovis* infection or where these programmes are not economically or socially acceptable. There is renewed interest in the use of TB vaccines for cattle stemming from the realisation of the financial impact of bovine TB on animal health and trade and also due to the difficulty of controlling the disease. Currently, there are no TB vaccines licenced for use in cattle, although the human TB vaccine, bacille Calmette-Guérin *M. bovis* (BCG) vaccine has been shown to induce significant levels of protection in cattle in experimental challenge and field trials (reviewed in [1,3]).

The major caveats which have restricted BCG being used in cattle until now have been that vaccination sensitises animals to respond in routine TB diagnostic tests, particularly in the first year after vaccination [4,5] and protection may not be complete [1,3]. Research has recently shown that the problem of BCG vaccination compromising conventional bovine TB diagnostic tests can be overcome by using tests which differentiate infected from vaccinated animals (DIVA tests), utilising specific mycobacterial antigens which are expressed by *M. bovis*, but not by BCG [6,7]. Secondly, protection can be enhanced by revaccinating with BCG when immunity has waned [8] or priming cattle with BCG and boosting with a sub-unit vaccine [9]. Registration of BCG vaccine for cattle will require extensive testing in the field as well as an assurance of safety for use of BCG vaccine in cattle, including the effect of vaccination of animals with a pre-existing *M. bovis* infection.

The effect of administration of mycobacterial preparations on an existing *M. bovis* infection in cattle is not documented, although insights can be gained from studies in humans and small animal models. Studies by Koch in the late 19th century revealed that immunisation of humans with a strong immunogen such as "old tuberculin", a glycerin filtrate of cultures of the tubercle bacillus, resulted in the exacerbation of the disease

leading to severe toxicities and worsening of the disease, a reaction now known as the "Koch phenomenon" [10]. Further, it is established that vaccination of humans or small animal models with BCG does not have a therapeutic effect on an existing *M. tuberculosis* infection [11,12], but a question remains whether BCG vaccine, could exacerbate an existing mycobacterial infection. It has been proposed that BCG vaccination of immunocompetent *M. tuberculosis*-infected individuals may result in increased reactogenicity and morbidity in latently-infected persons (Koch phenomenon) [13,14].

A dose of lyophilised BCG Danish vaccine, equivalent to five human doses (1-4 X 10^6 colony forming units, CFU), has commonly been used in TB vaccine efficacy trials for cattle, [9,15], although a 10-fold lower dose is still protective [16]. The aim of the current study was principally to test for the safety of administering a relatively high dose of BCG to cattle with a pre-existing *M. bovis* infection and a 4-fold variation in viable bacilli can be contained in a commercial human BCG vaccine dose (BCG Danish, Statens Serum institute, Copenhagen, Denmark). One group of cattle were vaccinated with the standard cattle dose of BCG at 21 weeks prior to challenge with *M. bovis* and a second group were vaccinated with a 4-fold higher dose of BCG vaccine at 3 weeks after challenge.

2. Materials & methods

2.1. Animals

Thirty-six Friesian-cross, male-castrated calves, 5-6 months old were obtained from herds which were accredited as TB-free for the previous 5 years and from an area of New Zealand where both farmed and feral animals were free of TB. Prior to the studies, the cattle tested negative for bovine TB in the whole blood IFN-γ test. The cattle were grazed on pasture in a biocontainment unit.

2.2 . *Bacterial strains and vaccines*

The lyophilised *M. bovis* BCG Danish 1331 vaccine (Statens Serum Institute, Copenhagen, Denmark) formulated for humans was utilised to vaccinate the calves. *M. bovis* WAg202, originally isolated from a tuberculous possum in New Zealand, was used as the challenge strain and had been used in previous vaccination/challenge studies in cattle [17,18]. Bacteria were grown to mid-log phase in Tween albumin broth (Dubos broth base, Difco Laboratories, Detroit, Mich.) supplemented with 0.006% (vol/vol) alkalinised oleic acid, 0.5% (wt/vol) albumin fraction V and 0.25% (wt/vol) glucose. Dilutions were made in Tween albumin broth to obtain the dose for inoculation. The number of CFU inoculated was determined retrospectively by plating 10-fold dilutions on Middlebrook 7H11 (Difco) supplemented with 0.5% (wt/vol) albumin, 0.2% (wt/vol) glucose and 1% (wt/vol) sodium pyruvate.

2.3 *Vaccination and M. bovis challenge*

The calves were divided into three groups, each containing 12 calves using a randomised stratified sampling system so that all groups contained animals with a similar distribution of IFN-γ responses to avian purified protein derivative (PPD; prepared from a *M. avium* culture) in the weeks prior to the start of the study. Calves from one group (BCG-vaccinated group) were each vaccinated subcutaneously in the left side of the neck with 0.5 ml of BCG vaccine (equivalent to 1.5 X 10^6 CFU/dose, with the other two groups remaining non-vaccinated. Each vial of BCG vaccine was reconstituted in 1 ml Sauton medium (Statens Serum Institute) and contained an estimated 2-8 X 10^6 CFU/vial, with retrospective culturing providing a count of 3 X 10^6 CFU/vial. All three groups of

calves were challenged endobronchially with 5 X 10^3 CFU of virulent *M. bovis* as previously described [17] at 21 weeks after vaccination. Three weeks after the *M. bovis* challenge, calves in one of the previously non-vaccinated groups (Post-challenge BCG group) were each vaccinated subcutaneously in the left side of the neck with 2 ml of BCG vaccine (6 X 10^6 CFU; contents of two vaccine vials) The remaining group was named the Non-vaccinated group.

2.4. Necropsy procedure

All cattle were killed 13 weeks after challenge. Procedures for identifying macroscopic tuberculous lesions and processing for histopathology have been described previously [16]. A lung lesion score was calculated by counting the total number of lesions and applying a score as follows: 0, no lesions; 1, 1-9 lesions; 2, 10-29 lesions; 3, 30-99 lesions; 4, 100-199 lesions; 5, ≥ 200 lesions. A total lymph node lesion score per animal was calculated by pooling scores for four major pulmonary lymph nodes (left bronchial and right bronchial/tracheobronchial and anterior and posterior mediastinal). Scores for individual lymph nodes were: 0, no lesions; 1, 1-19 small lesions (1-3 mm diameter); 2, ≥ 20 small lesions or medium size lesion (4-6 mm diameter); 3, large lesion (>6 mm). Samples from four pulmonary lymph nodes were collected from all of the animals for histology and bacterial culture. Additional samples were collected from any tuberculous-like lesions observed in lungs, other lymph nodes or organs. For histological examination, sections were stained with hematoxylin and eosin. Scoring of histopathological lesions for the four pulmonary lymph nodes was based on the scale of stage I to IV granulomas as described by Wangoo et al. [19]. Briefly, stage I granulomas were composed of accumulations of epitheloid macrophages with low numbers of lymphocytes, neutrophils and Langhans multinucleated giant cells and there was an absence of necrosis. Stage II granulomas were similar to stage I granulomas but also had central infiltrates of neutrophils and lymphocytes and necrosis could be present. Stage III granulomas exhibited complete fibrous encapsulation and significant necrosis and mineralisation could be present. Stage IV granulomas were characterised by multiple coalescing caseo-necrotic granulomas with multicentric necrosis and mineralisation. The percentage of the granulomas classified as Stage I, II, III or IV was calculated from the total number of granulomas for each group. Scoring of gross and histopathological lesions was undertaken blinded for animal number and treatment groups. For bacterial

culture, tissue samples (2-3 g) were homogenised in a Tenbroeck grinder (Wheaton, Millville N.J.), decontaminated in 0.75% cetylpyridinium chloride for 1 h, centrifuged at 3500 g for 20 min (included in the decontamination time) and processed for isolation of mycobacteria as described previously [17]. The CFU/g for each of the four pulmonary lymph nodes was determined and when no *M. bovis* was isolated from a sample, a value of half the minimal count was applied (5 CFU/sample) as not all the sample was cultured. The value for each animal was the mean of the $\log_{10}$ CFU/g of tissue for the four lymph nodes and the mean for the group calculated from these values. To measure cytokine mRNA expression, a small tissue sample from the left bronchial lymph node was collected from each animal and stored in RNAlater® (Life Technologies, USA). In the absence of a lesion in this lymph node, but if a lesion was identified in another pulmonary lymph node, the other lymph node was selected, otherwise a sample from the non-lesioned left bronchial node was selected.

2.5. *IFN-γ assay*

Heparinised blood samples were collected from the calves at regular intervals to analyse cellular immune responses. Blood samples (1.5 ml) were dispersed into wells of a 24-well plate and preservative-free bovine PPD prepared from a *M. bovis* culture or avian PPD (24 μg/ml final concentration; Prionics, Schlieren-Zurich, Switzerland) or phosphate-buffered saline (PBS, negative control) was added. Blood cultures were set up within 6 hours following blood collection. After incubation at 37^{o}C for 24 h, the plasma supernatants were harvested and their IFN-γ levels measured using a sandwich ELISA kit (Mabtech, Sweden). Results were reported as optical density units 450 nm (OD_{450}) for bovine or avian PPD minus OD_{450} for PBS.

2.6. Tuberculin skin test

The comparative cervical tuberculin skin test was undertaken at 10 weeks post-challenge. For this test, cattle were inoculated intradermally with 0.1 ml volumes containing either 3,000 IU of bovine PPD or 2,500 international units (IU) of avian PPD (Prionics, Lelystad, The Netherlands) at separate sites on the right side of the neck. The skin-fold thickness was measured with callipers prior to injection and 72 h after injection of the PPDs.

197 *2.7. Reverse-transcription and qPCR*

198 RNA was extracted from lymph node samples, purified and transcribed to cDNA as
199 previously described [20]. All cDNA samples were stored at −20°C until the qRT-
200 PCRwas undertaken. The primer sequences for IFN-γ, IL-10, IL-12p40, IL-17A,
201 interferon releasing factor-5 (IRF-5), iNOs and TNF-α were described by Shu et al. [21].
202 The forward and reverse primers for IL-2 were AACGGTGCACCTACTTCAAGCTCT
203 and TAGCGTTAACCTTGGGCGCGTAAA, respectively, while the corresponding
204 primers for CXCL9 were ACTGGAGTTCAAGGAGTTCCAGCA and
205 TCTCACAAGAAGGGCTTGGAGCAA and those for CXCL10 were
206 TCCTCGAACACGGAAAGAGGCATA and AGCTGATATGGTGACTGGCTTGGT.
207 For the qRT-PCR analysis, 10 µl of SyBr®Premix Ex Taq™ II master mixture (Takara
208 Bio Inc., Japan), 2 µl of template cDNA and 1 µl of 5 µM of each gene-specific primers
209 were combined in a 20 µl reaction mixture in duplicate. The amplification was performed
210 in a Rotor-Gene 6000 machine (Corbett Research, Australia). The cycle number at which
211 the various transcripts became detectable was referred to as the threshold cycle (Ct) and
212 data were analysed using Rotor-gene 6000 series software 7.0. The average Ct value of
213 duplicates was used for calculation of the relative fold changes using the ΔΔCt method
214 [22]. A previous study showed that the Ct values of the PCR with three house-keeping
215 genes, GAPDH, β-actin and U1 were consistent within each gene and U1 showed the
216 lowest Ct value [21]. We used U1 as the house keeping gene for normalisation and the
217 ΔCt from a pool of non-lesioned, prescapular lymph nodes from *M. bovis*-infected cattle
218 sourced from a previous study [21] was used as the calibrator to generate ΔΔCt.
219
220 *2.8. Statistical analyses*
221 For analysis of IFN-γ responses a mixed effects model was applied to natural log-
222 transformed IFN-γ responses; time, group and their interaction were fixed effects, and
223 animal and challenge (an indication variable for identifying before or after challenge)
224 were random effects. The Kruskal-Wallis test with multiple comparisons was used for
225 analysing lesion scores and qPCR data. Multiple comparisons of the different groups
226 were performed with a p-value adjusted by the 'BH' method [23]. These analyses were
227 undertaken using the R packages 'nlme', 'lme4' and 'predictmeans' in R 3.2.0 [24]. The
228 χ^2 test was used for comparing the distribution of the different granulomas stages for each
229 group. Fisher's Exact test was used for comparing the proportion of animals with lung or

lymph node lesions. For the remaining data, statistical analyses were undertaken using Minitab 16. The mean skin test values, numbers of lesioned lymph nodes/animal and *M. bovis* culture positive lymph nodes/animal as well as the mean $\log_{10}$ CFU/g from lymph nodes were compared using ANOVA with Tukey's multiple comparisons. Statistical significance was denoted when $P < 0.05$.

3. Results

3.1. Pathological and microbiological findings following M. bovis challenge

Vaccination of calves with BCG prior to *M. bovis* challenge (BCG-vaccinated group) produced a significant degree of protection against the challenge in one gross pathology parameter, with a lower median lymph node lesion score in the BCG-vaccinated group compared to those for the Non-vaccinated group (P <0.05, Figure 1A). In addition, BCG vaccination prior to challenge resulted significant reductions in the proportions of animals with lymph node and lung lesions, lower median lymph node and lung lesion scores and lower mean number of lesioned lymph nodes per animal compared to those for the Post-challenge BCG group (Table 1 and Figure 1A and B; $P < 0.05$). There were no significant differences between the gross pathology parameters for the Post-challenge BCG and Non-vaccinated groups. The lesions were typical of those for bovine TB with multiple small (1-3 mm in diameter) calcified lesions in the lung and variable sized calcified lesions in the pulmonary lymph nodes (1-20 mm in diameter). The number of animals in the BCG-vaccinated, Post-challenge BCG and Non-vaccinated groups with gross tuberculous lesions were 7, 12 and 10, respectively. No gross tuberculous lesions were observed outside of the pulmonary cavity.

Following histopathological examination, a comparison of the relative distribution of granuloma developmental stages was undertaken. This analysis revealed that the distribution of granuloma stages was significantly unequal between the three groups (Figure 2; $P = 0.0145$, χ^2 test). This was characterized by higher percentages of the most severe lesions (Stage IV) in the Post-challenge BCG and Non-vaccinated groups, and lower proportions of the less severe Stage 2 granulomata, compared to those for the BCG-vaccinated group of calves. The BCG-vaccinated and Non-vaccinated groups of animals had significantly lower mean numbers of pulmonary lymph nodes culture positive for *M. bovis* and lower mean $\log_{10}$ CFU of *M. bovis*/g of pulmonary lymph node than those for the Post-challenge BCG group (P <0.05, Table 1). No significant differences were detected between the BCG-vaccinated and Non-vaccinated groups.

No vaccination site reactions were observed following BCG vaccination in the Post-challenge BCG group.

3.2. IFN-γ responses after vaccination and challenge

The kinetics of T cell responses to *M. bovis* antigens were determined by measuring the release of IFN-γ from whole blood stimulated with bovine PPD (Figure 3A). Vaccination with BCG at the commencement of the study resulted in a significant increase in antigen-specific IFN-γ responses at 3, 6, 8, 10, 12 and 21 weeks after vaccination compared to the Non-vaccinated group ($P < 0.05$). Following challenge with *M. bovis* at 21 weeks post-vaccination, the mean IFN-γ responses for all groups increased, with the mean responses for the BCG-vaccinated and Post-challenge BCG groups significantly greater than that for the Non-vaccinated group at 3 weeks post-challenge ($P < 0.05$). The mean IFN-γ response for the Post-challenge BCG group was not boosted following vaccination with BCG at 3 weeks after challenge.

Although all the groups had a similar distribution of IFN-γ responses to avian PPD at 3 weeks prior to the start of the study, the mean responses for the Post-challenge BCG group were greater than those for the Non-vaccinated group at six of the seven time-points prior to challenge, although none of these differences were statistically significant (Figure 3B). In the period prior to challenge, there was a cumulative increase in the IFN-γ responses to both avian and bovine PPD in the Post-challenge BCG and Non-vaccinated groups which suggested exposure to environmental mycobacteria with the animals grazing on pasture.

3.3. Skin test responses after challenge

At 10 weeks after challenge with *M. bovis*, all animals with the exception of one animal from the Non-vaccinated group showed an increase in the skin fold thickness of > 1 mm at 72 hours following injection of bovine PPD. The only significant difference between the mean skin test responses was that the mean bovine PPD response for the Post-challenge BCG group (23.3 mm increase in skin fold thickness) was greater than that for the Non-vaccinated group (mean of 16.0 mm; Table 2; $P < 0.05$).

3.4. mRNA expression of immune mediators from pulmonary lymph nodes post-challenge

Tissue samples were collected from a pulmonary lymph node from each animal following slaughter of the animals 13 weeks after challenge to measure mRNA expression of immune mediators by qRT-PCR. Samples were preferentially selected from the left bronchial lymph node. Comparisons between the mean responses of immune

mediators are shown in Figure 4. The mean gene expression for IFN-γ, IRF-5, IL-12p40, IL-17A, iNOs, CXCL9, CXCL10 and TNF-α were significantly greater for the Post-challenge BCG group than those for the Non-vaccinated group ($P < 0.05$). In addition, the mean mRNA expression for IFN-γ, CXCL9 and CXCL10 were significantly greater for the Post-challenge BCG group than those for the BCG-vaccinated group ($P < 0.05$). No significant differences were detected between the groups for IL-2 and IL-10 mRNA expression, or between the BCG-vaccinated and Non-vaccinated groups for any of the immune mediators.

4. Discussion

There is increasing interest in the use of BCG vaccine to protect cattle against bovine TB, although it is recognized that similar to the situation in humans, BCG does not provide complete protection against TB at a population or individual animal level. In this study, a very stringent test was chosen to answer the question whether vaccinating infected cattle with BCG would modulate disease outcome. Cattle were vaccinated with a high dose of BCG only 3 weeks after a relatively high dose experimental challenge with *M. bovis*. Three weeks post-challenge is considered as an early stage of a *M. bovis* infection for cattle [25]. The dose of lyophilised BCG Danish vaccine most commonly administered subcutaneously to cattle in TB vaccine efficacy trials has been 0.05 to 0.5 ml (1-4 X 10^5 to 1-4 X 10^6 CFU/dose) [8,9,16] and for the current study, the 0.5 ml dose was chosen for immunisation prior to challenge. In the current study, this dose of BCG administered prior to challenge induced only minimal protection against TB with a significant lower median lymph node lesion score compared to the Non-vaccinated group. Marked variations in the efficacy of BCG vaccination have been previously reported for protection of cattle against experimental challenge with *M. bovis*, varying from a significant reduction in a single pathological or microbiological disease parameter [18] to a reduction in up to six parameters in a subsequent study [16]. The reasons for this variation are not clear, although prior sensitisation to environmental mycobacteria has been considered as a possible explanation for poor responses to BCG vaccination in cattle [26].

A 4-fold higher dose of BCG was chosen to vaccinate a group of previously non-vaccinated calves (Post-challenge BCG group) at 3 weeks post-challenge to test for safety due to the potential variation in the bacterial count that may be present in commercial BCG vaccines.. There was no significant difference in the gross pathology for the Post-challenge BCG and Non-vaccinated groups and both of these groups had a high percentage of the more advanced Stage IV granulomata compared to the pre-challenge BCG group. However, results from the mRNA expression of immune mediators indicated that there was a more severe inflammatory response at the site of infection in the pulmonary lymph nodes of the Post-challenge group compared to that for the Non-vaccinated group. qRT-PCR measurement of mRNA expression for eight of the 10 immune mediators from pulmonary lymph node tissues of the Post-challenge BCG group

was significantly greater than those for the Non-vaccinated group. Although, the colony counts of *M. bovis* in these lymph nodes were significant greater for the Post-challenge BCG group compared to that for Non-vaccinated group, the bacterial culture method did not allow virulent *M. bovis* to be differentiated from BCG. It is possible that BCG bacilli may have colonised the pulmonary lymph nodes in the Post-challenge BCG animals, contributing to the higher *M. bovis* counts.

Despite the limited availability of safety data for BCG vaccination of humans in high burden settings, no serious effects were reported following primary vaccination of tuberculin skin test positive persons in a large Indian trial [11]. Furthermore, BCG revaccination of latently infected adults with prior infant BCG vaccination was also shown to be safe and reactogenicity similar to that for primary BCG vaccination [27]. However, there are major differences in these trials compared to the current study. In the human TB trials, the infections were only defined as possible *M. tuberculosis* infections based on tuberculin skin test reactivity with the likelihood of non-specific mycobacterial or latent *M. tuberculosis* infections. In contrast, the *M. bovis* infection in the cattle resulted in a rapid development of tuberculous lesions in the non-vaccinated animals. It also needs to be stressed that the post-challenge vaccination took place very early after a severe *M. bovis* challenge at the height of the development of anti-tuberculous, cellular immune responses. Despite these severe experimental conditions, the post-challenge BCG did not lead to significant increase in gross and microscopic pathology.

A study in deer demonstrated that subcutaneous vaccination with 5 X 10^4 and 5 X 10^7 CFU of BCG Pasteur induced comparable levels of protection against infection and disease following intratonsillar challenge with *M. bovis*, [28]. In contrast, vaccination with a higher dose of 5 X 10^8 CFU of BCG Pasteur did not induce protection and evoked immune responses with a bias towards Type 2 rather than Type 1 reactivity. In the current study, there was no boosting of the whole blood antigen-specific IFN-γ responses following BCG vaccination for the Post-challenge BCG group. This may have been in part due to the enhanced reactivity to avian PPD antigens for this group in the period prior to challenge, resulting in a marked increase immediately post-challenge, masking any subsequent increase in the immune response following BCG vaccination. In comparison, vaccination with BCG prior to challenge (BCG-vaccinated group) using a 4-fold lower dose was shown to induce a sustained increase in the antigen-specific IFN-γ

response in the period, 3 to 21 weeks post-vaccination. The stronger tuberculin skin test response observed in the Post-challenge BCG group compared to that for the Non-vaccinated group was indicative of a stronger inflammatory response, possibly as a consequence of an enhanced reactogenicity following BCG vaccination post-challenge.

Studies in mice have provided information on possible detrimental effects of administering BCG following infection with *M. tuberculosis*. Although, BCG vaccination of mice prior to challenge with *M. tuberculosis* was protective, BCG vaccination of already infected mice did not improve the course of infection and repeated revaccination resulted in an exacerbation of the granulomatous response [12,29]. One of these studies showed that the increase in the lung tissue damage was associated with an increase in IL-17, TNF-α, IL-6 and MIP-2 expression and influx of granulocytes/neutrophils [12]. A pathological role for IL-17 was indicated as this response was abrogated in mice deficient in the gene encoding IL-23p19 or in the presence of IL-17 blocking antibody. In a further study, a single subcutaneous administration of live BCG to mice infected with *M. tuberculosis* increased antigen-specific T-cell proliferation and induced larger tuberculous lung granulomas, but did not induce a reduction in the bacterial load [30]. The authors suggested that an increased production of TNF-α resulting from vaccination post-challenge contributed to the increased inflammation in the lungs and accelerated death.

It has been reported that following a mycobacterial infection, an equilibrium is established between mycobacteria and the host through the interaction of mycobacteria and macrophages in granulomas, maintained by the release of immune mediators [31]. Vaccination after challenge may disturb this equilibrium causing a heightened immune response in the lesions, particularly when the vaccine is administered at the height of anti-*M. bovis* effector immune response. Administration of a high dose BCG vaccine to calves only 3 weeks after the *M. bovis* challenge induced a pro-inflammatory immune response in the pulmonary lymph nodes at 13 weeks post-challenge. There was a significantly higher expression of IFN-γ, IRF-5, IL-12p40, IL-17A, , iNOs, CXCL9, CXCL10 and TNF-α compared to that for the Non-vaccinated group, although only the responses to IFN-γ, CXCL9 and CXCL10 were also higher in this group compared to the animals vaccinated with BCG before challenge. The sequence of events is likely to have been initiated by the induction of IRF-5, a “master regulator” of the pro-inflammatory

cytokines, which up-regulates expression of IL-6, IL-12, IL-17, IL-23, TNF-α, CXCL10 and type 1 IFNs [32]. Subsequent production of IFN-γ induces the production of chemokines, CXCL9 and CXCL10, attracting more T lymphocytes and monocytes into the granulomas [33,34]. Expression of IL-10, the anti-inflammatory cytokine which inhibits the activity of Th1 cells, NK cells and macrophages [35], was not significantly increased in the pulmonary lymph nodes of the Post-challenge BCG group. Although, pro-inflammatory cytokines play an important role in control of mycobacterial infections, the timing and balance of the cytokines will influence whether these responses support control of infection versus detrimental inflammatory responses..

5. Conclusion

A very stringent test was used to determine the effect of administering BCG vaccine post-challenge, with cattle vaccinated with a high dose of BCG only 3 weeks after experimental infection with *M. bovis*. Compared to the Non-vaccinated group, vaccination with BCG post-challenge did not lead to protection or, alternatively, to a significant increase in gross and histo-pathology, although there was an up-regulation of an array of pro-inflammatory immune mediators from pulmonary lymph node tissues samples. The strong systemic IFN-γ responses to avian PPD observed in the Post-challenge BCG group prior to challenge may have contributed to the enhanced pro-inflammatory immune responses in the pulmonary lymph nodes of these animals following challenge and BCG vaccination. However, it does suggest caution in the use of high doses of BCG vaccine for cattle, where there is a possibility that animals may be infected with *M. bovis* prior to vaccination.

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Author's contributions

Study conception and design: BMB, NAP, AH, RGH, HMV, DNW; Data acquisition, analysis and interpretation: BMB, DS, NAP, SS, AH, DNW; Drafting and revising the manuscript: BMB, DS, NAP, SS, AH, HMV, DNW; Final approval: BMB, DS, NAP, SS, AH, RGH, HMV, DNW.

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Competing interests: The authors declare that no competing interests exist.

Ethical approval: All animal procedures were approved by an independent animal ethics committee from the AgResearch Grasslands Research Centre.

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545

546 **Table 1.** Gross pathological and microbiological findings after *Mycobacterium bovis*
547 challenge.

Group	Proportion with		Mean ±SEM no. of lesioned PLNs/animal	Mean ±SEM no. of *M. bovis* positive PLNs/animal	Mean ±SEM log_{10} CFU of *M. bovis*/g of PLN
	PLN lesions	Lung lesions			
BCG-vaccinated	4/12*	6/12†	0.67† (±0.33)	1.97† (±0.29)	1.23† (±0.38)
Post-challenge BCG	11/12	11/12	2.25 (±0.35)	3.17 (±0.30)	2.34 (±0.48)
Non-vaccinated	10/12	7/12	1.5 (±0.34)	1.75† (±0.35)	1.42† (±0.46)

548 PLN Pulmonary lymph node (pulmonary lymph nodes were the only lymph nodes with
549 gross tuberculous lesions)* Significantly less than those for the Post-challenge BCG and
550 Non-vaccinated groups ($P < 0.05$)

551 † Significantly less than that for the Post-challenge BCG group ($P < 0.05$)

552

553 **Table 2.** Mean (± SEM) skin test responses for cattle at 10 weeks after M*ycobacterium*
554 *bovis* challenge.

Group	Bovine PPD	Avian PPD
BCG-vaccinated	20.0	6.2
	(±2.4)	(±1.1)
Post-challenge BCG	23.3*	6.3
	(±1.7)	(±0.8)
Non-vaccinated	16.0	4.7
	(±1.9)	(±0.8)

555 * Significantly greater than that for the Non-vaccinated group ($P < 0.05$)

556

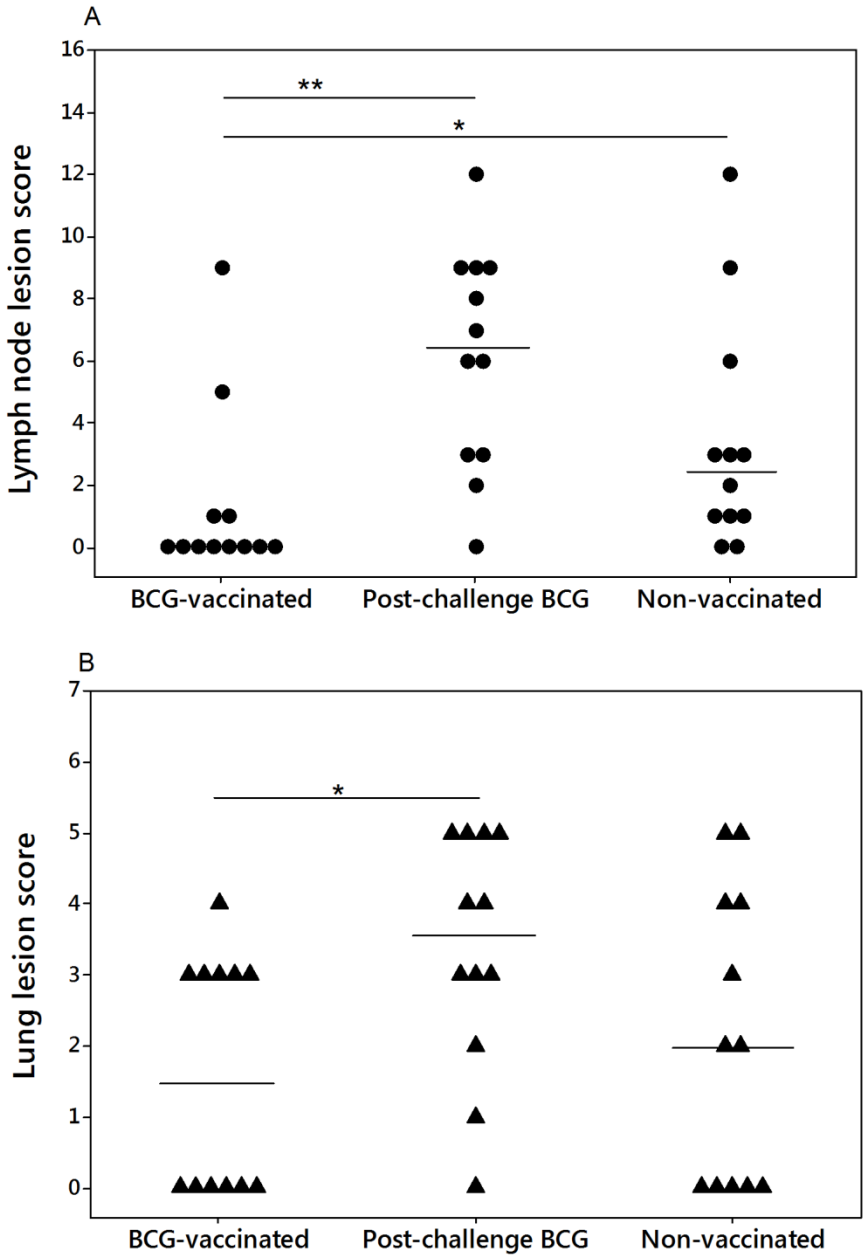
Figure 1. Lesion scores from the lymph nodes (A) and lung (B) for the BCG-vaccinated group (n=12); Post-challenge BCG group (n=12) and the Non-vaccinated group (n=12) after the *M. bovis* challenge. Total lymph node lesion score per animal: score for individual node: 0, no lesions; 1, 1-19 small lesions (1-3 mm diameter); 2, ≥ 20 small lesions or medium size lesion (4-6 mm diameter); 3, large lesion (>6 mm diameter), total lesion scores for four pulmonary lymph nodes pooled. Lung lesion score: 0, no lesions; 1, 1-9 lesions; 2, 10-29 lesions; 3, 30-99 lesions; 4, 100-199 lesions; 5, ≥ 200 lesions. Median indicated by horizontal line. Significant difference between groups, * $P < 0.05$, ** $P < 0.01$.

Figure 2. Percentages of the different granuloma stages in pulmonary lymph nodes of the BCG-vaccinated, Post-challenge BCG and Non-vaccinated groups. The histopathological granulomata stages (I, II, III and IV) are described in the Material and Methods. In total, 383, 952 and 391 granulomata were included in the analysis from BCG vaccinated, Post-challenge BCG treated and Non- vaccinated animals, respectively.

Figure 3. Mean IFN-γ responses following vaccination with BCG and *M. bovis* challenge. Figure 3 shows mean IFN-γ responses to bovine PPD (A) and avian PPD (B) from blood cultures reported as optical density units 450 nm (OD_{450}). BCG-vaccinated group (◆, n=12); Post-challenge BCG group (■, n=12) and the Non-vaccinated group (◇, n=12). Arrow V1 (Week 0) indicates vaccination for the BCG-vaccinated group; arrow C (Week 21) indicates *M. bovis* challenge for all groups; arrow V2 (Week 24) indicates vaccination for Post-challenge BCG group. Error bar represents SEM. Group mean was significant difference to that for the Non-vaccinated group was indicated by *, $P < 0.05$, with analyses performed on natural log-transformed data.

Figure 4. Relative mRNA expression of IFN-γ, IL-12p40, IL-2, CXCL9, IL-10, IRF-5, IL-17A, TNF-α, IL-10, CXCL10 and iNOs from pulmonary lymph nodes of the BCG-vaccinated group (BCG, n=12); Post-challenge BCG group (PC-BCG, n=12) and the Non-vaccinated group (NV, n=12). Target Ct values were normalised to U1 and a pool of non-lesioned prescapular lymph nodes was used as calibrator. The results were presented as relative fold change of mRNA in a box and whisker plot, with median shown as a horizontal line. Significant difference between groups, * $P < 0.05$, ** $P < 0.01$.

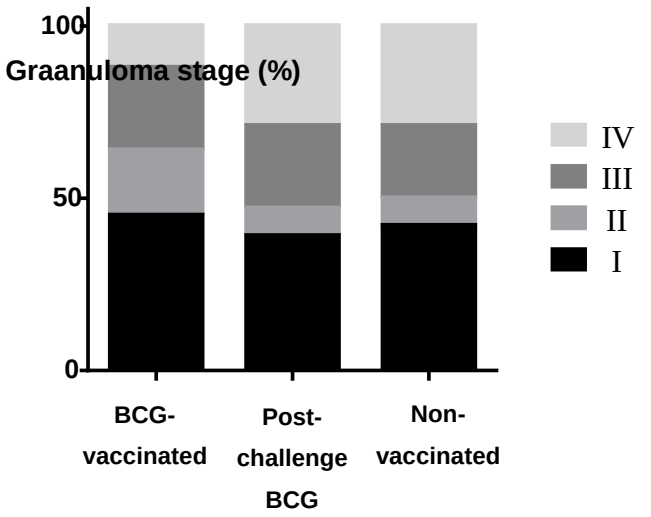
588 **Figure 1.**



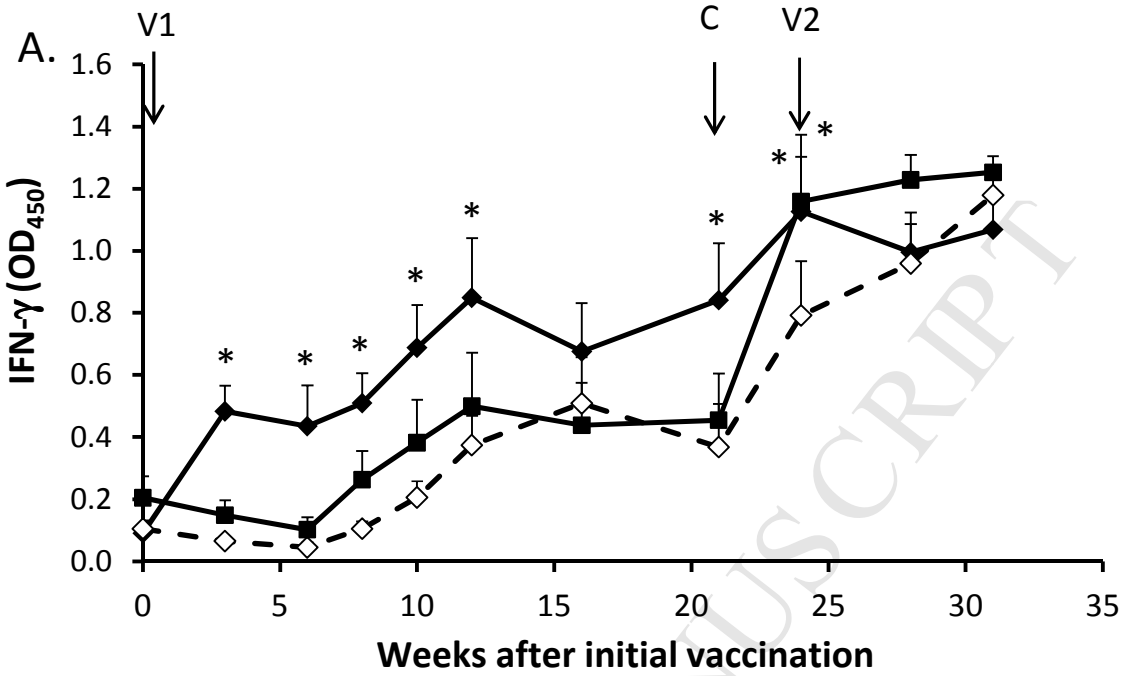
589

590 **Figure 2.**

591

592 

593 **Figure 3.**



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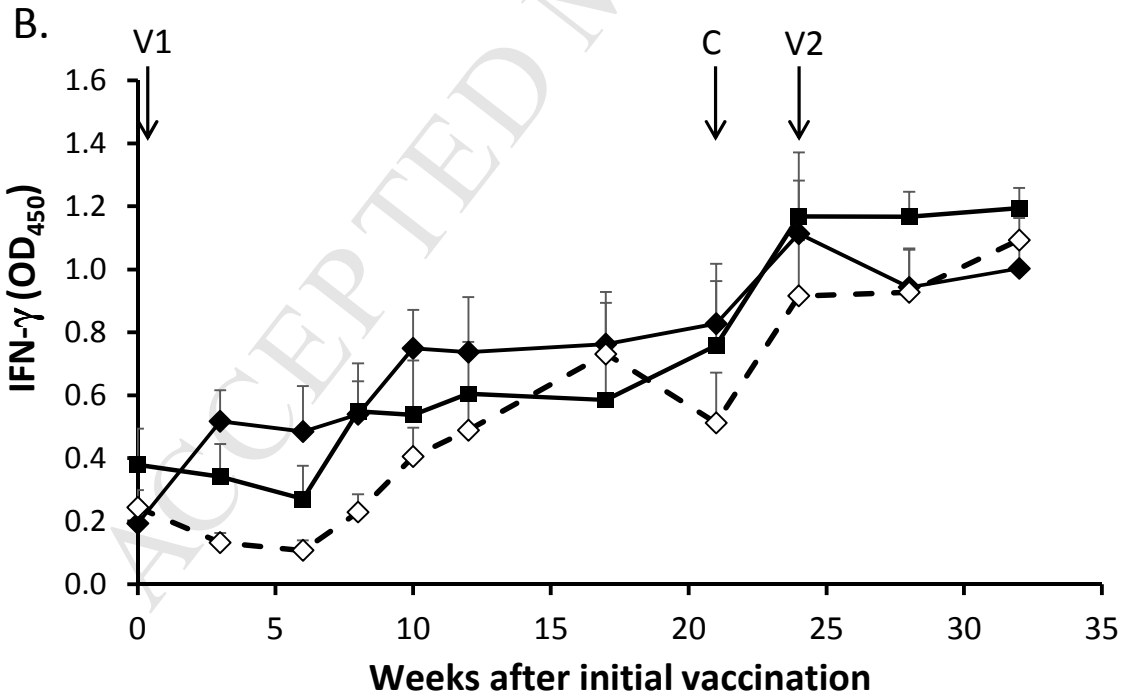

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597

598 **Figure 4.**

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600